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Endothelial Cells Assemble into a 3-Dimensional Prevascular Network in a Bone Tissue Engineering Construct

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ABSTRACT

To engineer tissues with clinically relevant dimensions, one must overcome the challenge of rapidly creating functional blood vessels to supply cells with oxygen and nutrients and to remove waste products. We tested the hypothesis that endothelial cells, cocultured with osteoprogenitor cells, can organize into a prevascular network *in vitro*. When cultured in a spheroid coculture model with human mesenchymal stem cells, human umbilical vein endothelial cells (HUVECs) form a 3-dimensional prevascular network within 10 days of *in vitro* culture. The formation of the prevascular network was promoted by seeding 2% or fewer HUVECs. Moreover, the addition of endothelial cells resulted in a 4-fold upregulation of the osteogenic marker alkaline phosphatase. The addition of mouse embryonic fibroblasts did not result in stabilization of the prevascular network. Upon implantation, the prevascular network developed further and structures including lumen could be seen regularly. However, anastomosis with the host vasculature was limited. We conclude that endothelial cells are able to form a 3-dimensional (3D) prevascular network *in vitro* in a bone tissue engineering setting. This finding is a strong indication that *in vitro* prevascularization is a promising strategy to improve implant vascularization in bone tissue engineering.

INTRODUCTION

VASCULARIZATION IS A CRITICAL PROCESS during bone growth. The suppression of blood vessel invasion results in thickening of the growth plate and impaired trabecular bone formation.[1] Apart from bone growth, vascularization is also involved in bone healing, both natural and in artificial bone implants. Studies have shown that fracture healing and ectopic new bone formation can be blocked by the administration of angiogenesis inhibitors,[2,3] while other studies have shown that new bone formation in porous scaffolds was significantly increased by the insertion of a vascular pedicle in the scaffold.[4,5]

To date, most approaches in tissue engineering have relied on vascularization by the ingrowth of blood vessels from the host. After implantation of tissue constructs, the supply of oxygen and nutrients to the implant is limited by diffusion processes and the speed of ingrowth of host vessels. In active tissue, sufficient diffusion is confined to 150 μm from the next capillary,[6] and the formation of host vessels within the construct takes time.[7] This leads to nutrient limitations and/or hypoxia. Moreover, nutrient and oxygen gradients will be present in the outer regions of the scaffold,[8] which could result in nonuniform cell differentiation and integration. If tissue engineering is ever to routinely surpass the tissue thickness limit of 100–200 μm, it must overcome the challenge of creating functional blood vessels to supply cells with oxygen and nutrients and to remove waste products.[9]

In the field of bone tissue engineering, bone implant materials such as ceramics are combined with osteoprogenitor cells. Human mesenchymal stem cells (hMSCs) are commonly used as a source for osteoprogenitor cells. These pluripotent cells are isolated from the bone marrow and have the ability to differentiate into adipogenic, chondrogenic, and osteogenic lineages.[10] Differentiation toward osteoprogenitor

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cells can be achieved by stimulation with, for instance, bone morphogenic protein-2 or dexamethasone.[11–13] Numerous groups, including our own, have shown that the combination of artificial scaffolds and osteoprogenitor cells can lead to the formation of new bone in both ectopic and orthotopic sites.[14–21] Although the mechanism of bone formation in this setting is not yet fully understood, there is evidence that the implantation of osteoprogenitor cells affects bone formation only if the cells are viable,[21] suggesting that the implanted cells play an active role in the formation of new bone. Vascularization is therefore not only necessary for new bone formation; it is also vital for the survival of the implanted cells on the carrier material after implantation.

A strategy to enhance the vascularization of an implant is the delivery of one or more angiogenic molecules directly to the site of interest. Such molecules include, but are not limited to, vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), and transforming growth factor-β.[9] Indeed, the dual delivery of VEGF and PDGF-BB from a polymer scaffold resulted in the formation of a mature vascular network.[22] However, this technique still relies on the ingrowth of host endothelial and mural cells, and therefore the vascularization will still take considerable time. So, even though the dual delivery of VEGF and PDGF-BB does show a significant increase in vascularization after 2 weeks, it remains uncertain whether an effect is present at earlier time points, which are most crucial for cell survival.

An alternative approach could be the vascularization of engineered tissue constructs before implantation. Endothelial cells, cultured with or without other cell types on scaffolds or in gels, can spontaneously form a capillary-like network *in vitro*.[23–26] Moreover, we recently showed that a prevascular network formed *in vitro* in a muscle construct anastomosed to the host vessels after implantation, resulting in a better vascularization and survival of the implant tissue.[27] Since this approach does not rely on the ingrowth of host endothelial cells into the entire construct but rather only into the outer regions, it may result in much faster vascularization of the implant.

The goal of the current study was to create an endothelial network in a bone tissue engineering construct. We hypothesized that endothelial cells cocultured with osteoprogenitor cells can organize into a prevascular network *in vitro*. Such a network may contribute to the early vascularization of the implant *in vivo*, resulting in better survival of the implanted cells and enhanced bone formation.

MATERIALS AND METHODS

Culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (East Rutherford, NJ). Cells were grown at 37°C in a humid atmosphere with 5% carbon dioxide (CO_2) in endothelial growth medium-2 (Cambrex). Cells were routinely split at a 1:5 ratio and cultured in fewer than 5 passages. Only HUVECs from passage 3 or 4 were used to seed the coculture experiments.

Isolation and culture of human mesenchymal stem cells

Bone marrow aspirates (5–20 mL) were obtained from 3 donors, age 36, 43, and 49 years, with written informed consent. Human mesenchymal stem cells (hMSCs) were isolated and proliferated as described elsewhere.[28] Briefly, aspirates were resuspended by using a 20-gauge needle, plated at a density of 5×10^5 cells/cm^2, and cultured in minimal essential medium (α-MEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (AsAP, Sigma, St. Louis, Missouri), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin (Invitrogen), 10 μg/mL streptomycin (Invitrogen), and 1 ng/mL basic fibroblast growth factor (bFGF, Instruchemie, Delfzijl, the Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Cells were routinely split at a 1:5 ratio and cultured in fewer than 5 passages. hMSCs from passage 3 or 4 were used to seed the coculture experiments.

Culture of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were purchased from Cellartis (Göteborg, Sweden). Cells were grown at 37°C in a humid atmosphere with 5% CO_2 in Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Cambrex), 100 U/mL penicillin (Invitrogen), and 10 μg/mL streptomycin (Invitrogen). Cells were routinely split at a 1:5 ratio.

Proliferation of HUVECs and hMSCs in different media

To assess the proliferation of HUVECs and hMSCs in different media, they were seeded in T25 culture flasks at a density of 2.700 cells/cm^2 and 2.000 cells/cm^2, respectively. Cells were cultured for 4 days in 3 different media: 1) HUVEC medium (EM) (EBM-2, Cambrex), 2) osteogenic differentiation medium (ODM) (α-MEM supplemented with 10% FBS, 0.2 mM AsAP, 2 mM L-glutamine, 100 U/mL penicillin, 10 μg/mL streptomycin, 10^{-8} M dexamethasone [Sigma], and 0.01 M β-glycerophosphate [Sigma]), and 3) a 1:1 mix of the first 2 media (EODM). Each day, the cells of 3 flasks were counted by using a Coulter counter (Beckman Coulter, Fullerton, California).

HUVECs and hMSCs were cocultured without direct contact in ODM by using cell culture inserts (Becton-Dickinson, Franklin Lakes, NJ). HUVECs were seeded at

a density of 2.700 cells/cm^2 in the wells and hMSCs at a density of 2.700 cells/cm^2 in the cell culture inserts. Cells were allowed to proliferate for 3 days, after which pictures were taken to assess the proliferation of the HUVECs.

Generation and culture of HUVEC-hMSC coculture spheroids

Different percentages of HUVEC and hMSC were pooled to a total of 5×10^5 cells in a round-bottom 10-mL tube (Greiner, Longwood, FL). The cells were resuspended in 4.5 mL of ODM and consequently centrifuged at 1700 rpm for 2 min. The tubes with the cell pellets were incubated at 37°C in a humid atmosphere with 5% CO_2, which allowed for the spontaneous formation of coculture spheroids. The spheroids were either cultured for 3 days without a medium change, or for 10 days with a medium change at day 5 and day 8.

In vivo organization assay

After 10 days of *in vitro* culture, 4 spheroids seeded with 2% HUVECs and 98% hMSCs were implanted subcutaneously in the dorsal region of 2 male nude mice. The mice were anesthetized with 2.5% isoflurane, after which the spheroids were implanted in separate pockets. Two weeks after implantation, lectin *Helix pomatia* agglutinin conjugated to Alexa Fluor 594 (Invitrogen) (0.5 mg/0.25 mL phosphate-buffered saline [PBS]) was injected into the tail vein of anesthetized animals (20 mg/kg body weight). Circulation was allowed for 2 min, after which the animals were euthanized and the implants were retrieved. Samples were snap-frozen in Cryomatrix (Thermo Shandon, Waltham, MA).

Immunohistochemical analysis

After harvesting, spheroids were frozen in Cryomatrix at −60°C. Sections (6 μm) were cut with a cryotome. Sections were fixed in cold acetone for 5 min and air-dried. Sections were rehydrated for 10 min, after which they were incubated for 30 min with 10% FBS in PBS to block nonspecific background staining. Sections were incubated with mouse-anti-human CD31 (which does not crossreact with mouse tissue) or mouse-anti-human smooth muscle actin (which does crossreact with mouse tissue) primary antibody (Dako, Glostrup, Denmark) for 1 h. Sections were washed in PBS and subsequently incubated with the secondary antibody (horseradish peroxidase conjugated goat-anti-mouse immunoglobulin antibody, Dako) for 45 min. Slides were developed with diaminobenzidine (Dako) as substrate and were weakly counterstained with hematoxylin (Sigma). For the *in vivo* samples, Alexa Fluor 488 conjugated goat-anti-mouse immunoglobulin antibody (Invitrogen) was used as the secondary antibody. These samples were neither developed nor counterstained.

Whole spheroids were fixed in cold acetone (−20°C) for 6 min and subsequently rehydrated in tap water for 15 min. Spheroids were incubated in 10% FBS in PBS for 90 min to block nonspecific background staining. Spheroids were incubated with mouse-anti-human CD31 primary antibody (Dako) for 2 h. Spheroids were washed in PBS for 1 hour and subsequently incubated with the secondary antibody (Alexa Fluor 488 conjugated goat-anti-mouse immunoglobulin antibody, Invitrogen) for 2 h.

Image analysis

Images of the entire surface of cross-sections were taken at a magnification of ×100, unless otherwise stated (Eclipse E600, Nikon, Tokyo, Japan). Images were combined to make a single image of the entire cross-section of each sample. Subsequently, CD31-positive areas were pseudo-colored and Bioquant Image Analysis software (Nashville, TN) was used to determine the percentage of the cross-section that stained positive for CD31. Statistical analysis was performed by using the Student *t*-test.

RNA isolation and quantitative polymerase chain reaction

Spheroids were seeded with 100% hMSCs or 95% hMSCs plus 5% HUVECs and subsequently cultured in osteogenic differentiation medium for 10 days *in vitro*. To acquire sufficient RNA per sample, 3 spheroids were pooled and fragmented mechanically. Three samples (9 spheroids) were prepared. Total RNA was isolated by using a RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) and on column DNase treated with 10U RNase free DNase I (Invitrogen) at 37°C for 30 min. DNAse was inactivated at 72°C for 15 min. The quality and quantity of RNA were analyzed by spectrophotometry. Two μg of RNA was used for first strand complementary DNA (cDNA) synthesis by using Superscript II (Invitrogen) according to the manufacturer's protocol. One μL of 100×diluted cDNA was used for collagen type I and 18s ribosomal RNA (rRNA) amplification, and 1 μL of undiluted cDNA was used for other genes. Polymerase chain reaction (PCR) was performed on a Light Cycler real-time PCR machine (Roche, Basel, Switzerland) by using SYBR Green I Master Mix (Invitrogen). Data were analyzed by using Light Cycler software version 3.5.3, using fit-point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of the alkaline phosphatase gene was calculated relative to 18s rRNA levels by the comparative ΔCT method.[28] Statistical analysis was performed by using the Student *t*-test. Primer sequences were as follows: human 18s rRNA, 5′-CGGCTACCACATCCAAGGAA-3′ and 5′- GCT GGAATTACCGCGGCT-3′; human alkaline phosphatase, 5′-GACCCTTGACCCCCACAAT-3′ and 5′-GCTCGTACT GCATGTCCCCT-3′.

RESULTS

Cell proliferation in different media

To test the behavior of both HUVECs and hMSCs in different media, they were cultured on tissue culture plastic in 3 different media: EM, ODM, and EODM. The hMSCs proliferated well on tissue culture plastic in all 3 media. Surprisingly, proliferation was highest when hMSCs were cultured in EODM. Proliferation was lowest in ODM, and EM gave an intermediate result. Although proliferation was highest in EODM, the morphology of the cells also changed in this medium (data not shown), indicating that EM influences the differentiation of hMSCs. HUVECs proliferated well on tissue culture plastic when it was cultured in EM. When cultured in ODM, however, HUVECs did not proliferate. When cultured in the mixed medium, HUVECs proliferated, but growth rate was reduced (Fig. 1). To study whether growth factors secreted by hMSCs could help HUVECs to proliferate in osteogenic medium, HUVECs were cocultured with hMSCs without direct contact in a cell culture insert assay in ODM. Even though this system allowed the exchange of growth factors between the 2 cell types, no proliferation of the HUVEC cells could be detected after 3 days.

Spontaneous formation of coculture spheroids

To study the organization of the endothelial cells in a dense 3D cellular environment, a spheroid coculture method was developed. Solid spheroids with a diameter of approximately 1 mm were formed spontaneously between days 3 and 5 with the described method. The spheroids remained intact for the rest of the 10-day culture period (Fig. 2A). Although most cells were incorporated in the spheroids with the method used, smaller cell aggregates were also detected in the medium. The number of these aggregates varied from sample to sample and appeared to be independent of the test parameters. Bone marrow cells from 3 different donors were used to determine whether the formation of cell spheroids was universal or donor specific. The hMSCs of different donors gave similar results regarding spheroid formation (data not shown).

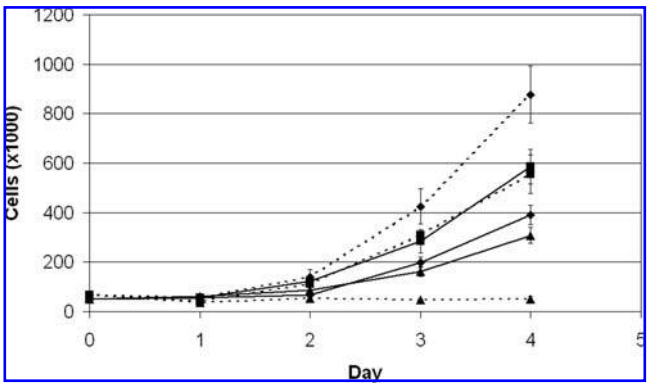

FIG. 1. Proliferation of human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs) in different media. The HUVECs and hMSCs were cultured in HUVEC medium, osteogenic medium, and a 1:1 mixed medium on tissue culture plastic for 4 days. The number of cells was determined every day. Solid line: hMSCs, dotted line: HUVECs, diamond: HUVEC medium, triangle: osteogenic medium, square: mixed medium. Results shown are mean values ± standard deviation ($n = 3$).

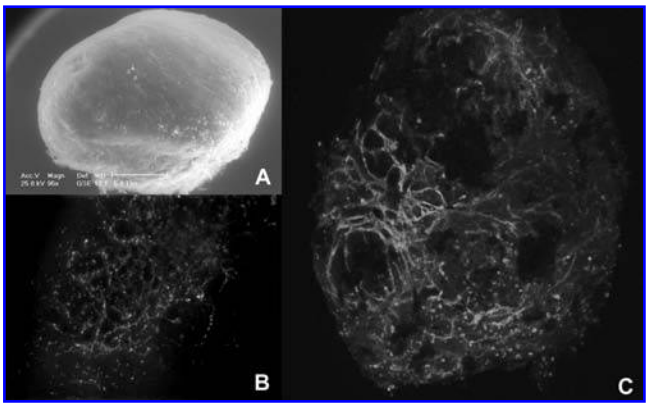

FIG. 2. (**A**) Scanning electron microscope picture of a coculture spheroids cultured *in vitro* for 10 days. Scale bar = 200 μm. (**B and C**) Entire spheroid immunostained with human-specific anti-CD31 antibodies, showing the formation of a 3-dimensional prevascular network. Images were made by using a conventional fluorescence microscope (**B**) or a convocal fluorescence microscope (**C**).

Organization of endothelial cells into a 3D prevascular network

After 3 days of pellet coculture, endothelial cells were present as round, individual cells throughout the pellets (data not shown). Image analysis showed that the percentage of endothelial cells had decreased between seeding and day 3 (Fig. 3). Cross-sections of spheroids cultured for 10 days showed that endothelial cells were still present throughout the spheroids, for all concentrations of HUVECs seeded (Fig. 4). Moreover, endothelial cells had organized into elongated, vessel-like structures. Pictures of whole cell spheroids confirmed that the structures connected to each other and formed a 3D prevascular network of endothelial cells (Fig. 2). Although endothelial cells did organize into a 3D prevascular network, the organization of the network was still primitive, as illustrated by the lack of lumen inside the structures (Fig. 4). Spheroids seeded with different percentages of endothelial cells showed a similar organization of endothelial structures up to 10% of HUVECs seeded. When higher percentages of HUVECs were used, structures appeared less elongated and more endothelial cells could be seen in cell clumps rather than vessel-like structures (Fig. 4). The area of cross-sections of the spheroids that stained positive for the endothelial marker CD31 was determined.

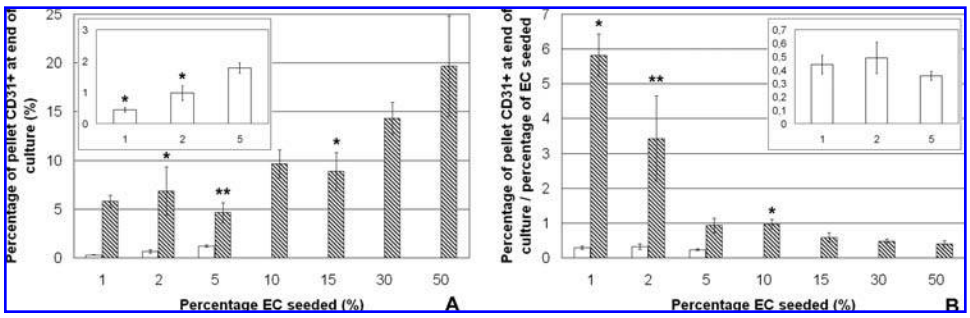

FIG. 3. Percentages of coculture spheroid cross-section that stain positive for the endothelial marker CD31. Coculture spheroids were seeded with different percentages of human umbilical vein endothelial cells (HUVECs) and cultured *in vitro* for 10 days. Results shown are mean values ± standard deviation. White bars and insets display the situation after 3 days of culture ($n = 4$), striped bars show the situation after 10 days of culture (1%: $n = 3$, 1 donor/2%: $n = 6$, 2 donors/5%: $n = 10$, 3 donors/10–50%: $n = 3$, 1 donor). (**A**) Percentage of cross-section that stains positive for CD31 after 3 or 10 days of *in vitro* culture plotted against the percentage of HUVECs seeded. (**B**) Relative expansion of CD31-positive cells during 3 or 10 days of *in vitro* culture plotted against the percentage of HUVECs seeded. $*p < .05$; $**p < .005$, compared with the group on the right side. EC: endothelial cell.

Positive staining was never seen in samples that were seeded with 0% HUVEC. For the samples that were seeded with 50% HUVEC, almost 20% of the cross-section stained positive for the CD31 marker. This percentage dropped with a decrease in the HUVEC seeding density. For samples that were seeded with 5% HUVEC, the area that stained positive for the marker was 5%. Interestingly, from that point on the percentage remained a rather constant 5% when the

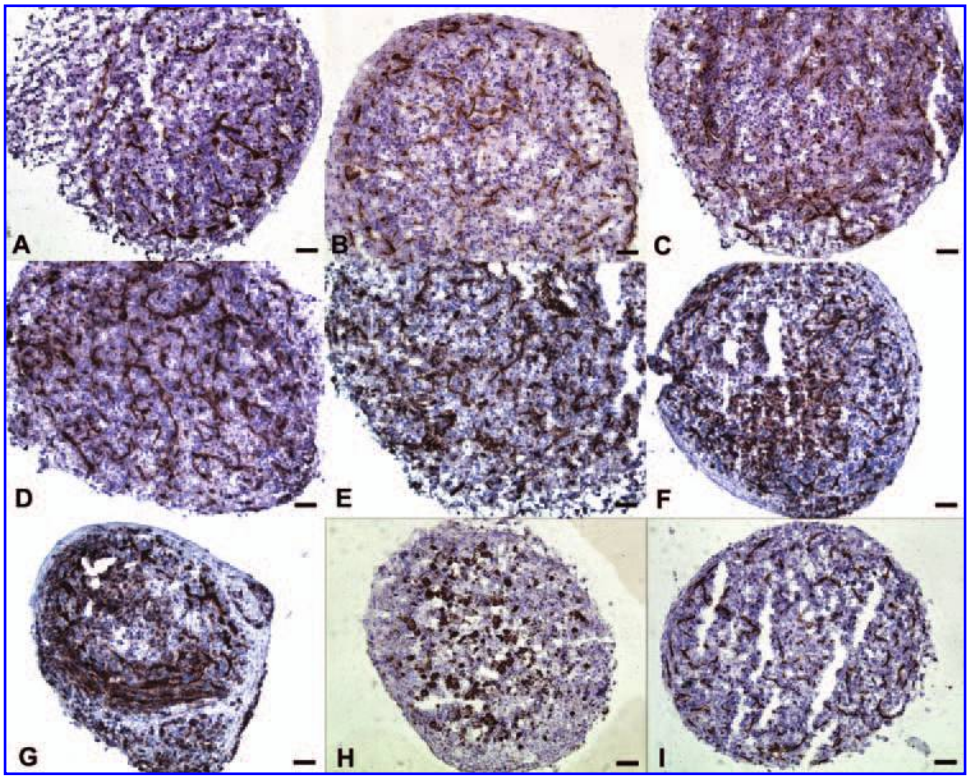

FIG. 4. Organization of endothelial cells in cocultures seeded with different percentages of human umbilical vein endothelial cells (HUVECs). Spheroids were cultured for 10 days *in vitro*, and cross-sections were immunostained with human-specific anti-CD31 antibodies (brown) showing the formation of vessel-like structures. Scale bar = 200 μm. (**A**) 1% HUVECs seeded. (**B**) 2% HUVECs seeded. (**C**) 5% HUVECs seeded. (**D**) 10% HUVECs seeded. (**E**) 15% HUVECs seeded. (**F**) 30% HUVECs seeded. (**G**) 50% HUVECs seeded. (**H and I**) Effect of mouse embryonic fibroblasts (MEFs) on the formation of the prevascular network. Spheroids were seeded with 85% hMSCs plus 5% HUVECs plus 10% MEFs and cultured *in vitro* for 10 days. Cross-sections were immunostained with human-specific anti-smooth muscle actin (**H**) and human-specific anti-CD31 (**I**) (brown). Colocalization of smooth muscle actin–positive cells and CD31-positive cells could not be detected. Scale bar = 100 μm. Color images available online at www.liebertpub.com/ten.

percentage of HUVECs seeded was decreased further up to 1% (Fig. 3A). This means that seeding low percentages of HUVECs has a stimulatory effect on the formation of the prevascular network.

This phenomenon is better demonstrated by plotting the relative expansion of CD31-positive cells against the percentage of HUVECs seeded (Fig. 3B). hMSCs from 3 different donors were used to determine whether the organization of the endothelial cells was universal or varies with the source of bone marrow cells used. Bone marrow cells of different donors gave similar results regarding endothelial cell organization and proliferation in the cocultures (data incorporated in Fig. 3).

Upregulation of alkaline phosphatase expression in coculture spheroids

The expression of the osteogenic marker alkaline phosphatase was determined in both coculture spheroids and hMSC spheroids. Quantitative PCR analysis showed that the expression of alkaline phosphatase is significantly ($p < .05$; $n = 3$) upregulated with a factor of 4 ± 1.7 by adding 5% HUVECs to the spheroids.

Effect of addition of MEFs on prevascular structures

MEFs were added to the cocultures to test whether these cells can differentiate toward smooth muscle cells and contribute to the stabilization of the prevascular network. When MEFs were added to the hMSC-HUVEC cocultures, smooth muscle actin could be detected in the spheroids, suggesting that cells in these tricultures differentiated toward smooth muscle cells (Fig. 4). Since this phenomenon could not be detected in cocultures of hMSCs and HUVECs alone, it suggests that either the MEF differentiated toward smooth muscle cells or helped other cells in the triculture to differentiate toward smooth muscle cells. Cells positive for smooth muscle actin were distributed throughout the spheroids, with the exception of the outer perimeter of the spheroids where only a few positive cells were detected. Cells positive for smooth muscle actin did not organize along the vessel-like endothelial structures, and there was no preferred colocalization with these structures. Although MEFs seemed to differentiate toward smooth muscle cells in this system, they did not contribute to stabilization of the vessel-like endothelial structures. This was further confirmed by the fact that triculture spheroids did not show an increase in the amount and size of vessel-like structures that were formed (data not shown).

Stability and organization of the prevascular network in vivo

Coculture spheroids cultured *in vitro* for 10 days were implanted subcutaneously in nude mice to assess the stability and potential anastomosis of the prevascular network *in vivo*. Because of migration of the samples or complete incorporation in the mouse tissue, only 3 of 4 spheroids could be retrieved after 2 weeks of implantation. Cross-sections of the retrieved spheroids confirmed that vessel-like structures were still present after 14 days of subcutaneous implantation. The structures had developed further, and lumen could now frequently be seen inside the vessel-like structures (Fig. 5). To determine whether the implanted prevascular network had

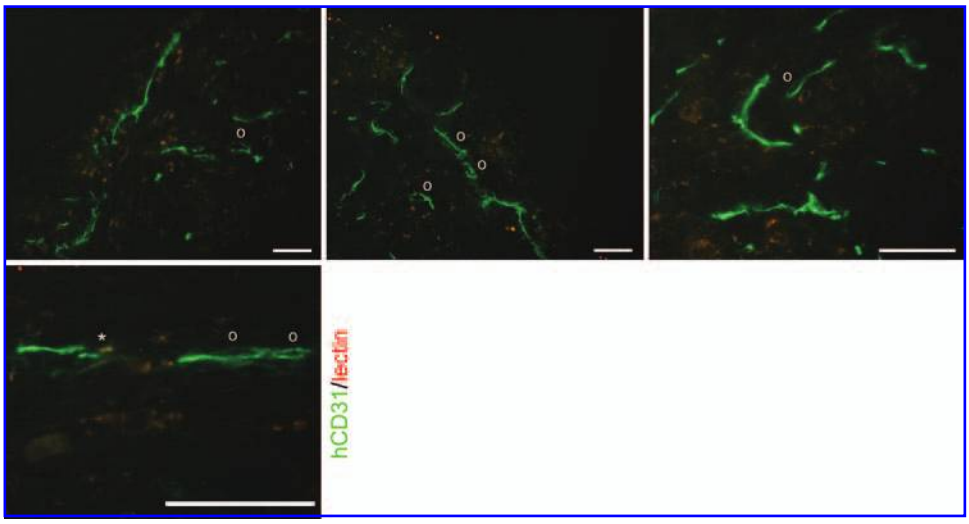

FIG. 5. *In vivo* analysis of coculture spheroids. Spheroids seeded with 2% human umbilical vein endothelial cells plus 98% human mesenchymal stem cells were cultured *in vitro* for 10 days and then implanted subcutaneously in the dorsal region of nude mice for 14 days. Labeled lectin was injected into the tail vein before explantation to asses perfusion of the implant (red). Cross-sections were immunostained with human-specific anti-CD31 antibodies (green). Note the presence of lumen in the vessel structures (o) and the local costaining of lectin and anti-human-CD31 (asterisk). Scale bar = 50 μm. Color images available online at www.liebertpub.com/ten.

anastomosed and become functional, we injected labeled lectin into the tail vein of the mice before explantation. Lectin perfusion showed that locally, there was costaining of human CD31 and labeled lectin (Fig. 5), indicating either that there was anastomosis of the human vessel-like structures with the blood system of the host or that single or multiple human endothelial cells were incorporated in remodeling or growing blood vessels of the host. Costaining was seen infrequently, only in small structures and only at the edge of the spheroids. No perfused human vessel structures could be detected farther away from the periphery of the implant.

DISCUSSION

Rapid vascularization is critical in most cell-based tissue engineering applications to ensure optimal cell survival and implant integration. Several strategies to improve vascularization have been investigated. Most strategies, however, rely on the ingrowth of blood vessels from the host, meaning that vascularization still takes considerable time. A potential strategy to circumvent this is to combine the implant with a prevascular network *in vitro* that can connect to the blood system of the host after implantation, resulting in the fast formation of a vasculature in the implant.

In this study, we investigated the hypothesis that an *in vitro* coculture strategy with osteoprogenitor cells and endothelial cells can result in a prevascular network for the application in bone tissue engineering. We showed that endothelial cells in the presented spheroid coculture model form a 3D prevascular network *in vitro*. The organization of the endothelial cells into a network was seen generally in all samples, but was promoted by seeding 2% HUVEC or less. Addition of MEFs did not result in stabilizing smooth muscle cells or in an increase in the amount of vessel-like structures. Upon implantation of the coculture spheroids, the prevascular network developed further and lumen could be seen regularly inside the vessel-like structures. Although there was evidence that human endothelial cells or structures in the periphery of the spheroids were incorporated in host vessels, the implanted prevascular network did not yet become extensively perfused.

To our knowledge, we report for the first time the formation of a 3D prevascular network combined with osteoprogenitor cells for the use in bone tissue engineering. Spheroidal cocultures of osteoprogenitor cells and endothelial cells have been reported before,[29,30] but the formation of a 3D prevascular network inside the spheroids had not been further addressed. In contrast, Stahl *et al.*[29] report that coculture spheroids differentiate spontaneously to organize into a core of osteoblasts and a surface layer of endothelial cells. The differences in endothelial cell organization may arise from the fact that these authors used only 500 cells per spheroid and performed the cocultures with a 1:1 ratio of osteoblasts and endothelial cells; as we have demonstrated, this is not an optimal ratio for endothelial cell organization. Why low percentages of endothelial cells positively affect the formation of the prevascular network remains unclear. After 3 days of culture, there was not yet a statistically relevant difference between the groups seeded with low percentages of HUVEC, indicating that this phenomenon is not caused by differences in seeding efficiencies. Most likely, the communication between the different cell types is more optimal when low percentages of HUVECs are seeded, allowing for a better proliferation and organization of these cells. However, the possibility that a subfraction of hMSCs differentiates toward endothelial cells and gets incorporated into the prevascular structures cannot yet be ruled out.

The hMSCs are commonly used as a source for osteoprogenitor cells. These pluripotent cells are isolated from the bone marrow and have the ability to differentiate into adipogenic, chondrogenic, and osteogenic lineages.[10] To stimulate osteogenic differentiation of the hMSCs, osteogenic medium was chosen as the coculture medium, even though HUVECs were unable to proliferate in this medium on cell culture plastic. Surprisingly, proliferation of HUVECs can be seen in this medium in the coculture spheroids. Previous studies have shown that hMSCs secrete growth factors that enhance endothelial cell proliferation.[31,32] However, an indirect coculture model of HUVECs and hMSCs in osteogenic differentiation medium could not restore the proliferation of endothelial cells. This suggests that the effect of the hMSCs on the proliferation of HUVECs in the coculture spheroid model occurs not only via the excretion of growth factors but also via direct cell-cell contact. This direct coupling between endothelial cells and osteoprogenitor cells has been reported before.[33]

Blood vessels are stabilized by association with pericytes or smooth muscle cells.[24,27,34,35] Moreover, endothelial cells can induce the differentiation of undifferentiated embryonic fibroblasts into smooth muscle cells.[24,36] We therefore hypothesized that the formation of vessel-like structures in the spheroid cocultures could be promoted by the addition of undifferentiated embryonic fibroblasts. The addition of MEFs to the spheroids, however, did not result in stabilization of the vessel-like structures, even though cells differentiated toward smooth muscle cells as indicated by the expression of smooth muscle actin. Whether the smooth muscle actin–positive cells arise from hMSCs or MEFs remains uncertain. It has been reported that hMSCs can express smooth muscle actin and that direct coculture with endothelial cells enhances the expression of smooth muscle actin by hMSCs.[37] With the staining techniques used in these studies, however, clear positive staining for smooth muscle actin was detected not in the coculture spheroids but rather only in the triculture spheroids, including the embryonic fibroblasts; this finding indicates that the embryonic fibroblasts give rise to the smooth muscle actin–positive cells. It is unclear why the smooth muscle actin–positive cells did not colocalize with or stabilize the endothelial structures.

Previous research has shown that human endothelial networks cultured *in vitro* can anastomose to the host vasculature within 2 weeks after implantation.[27,35] In the present study, however, integration of the prevascular network with the host vasculature was limited. It is uncertain whether this was due to insufficient differentiation of the prevascular network *in vitro*, the lack of stabilizing smooth muscle cells, or the potential presence of other factors that prevent anastomosis. Even though the present data do not show the formation of an extensive functional vascular network arising from the implanted endothelial cells, they do indicate that the prevascular network formed *in vitro* is stable after implantation and organizes further *in vivo*.

The combination of endothelial cells and osteoprogenitor cells could have beneficiary effects other than the acceleration of vascularization. Endothelial cells and osteoprogenitor cells are known to secrete an array of growth factors that are beneficial for the growth and differentiation of the other. Osteoprogenitor cells are known to secrete VEGF in quantities high enough to enhance the survival and differentiation of endothelial cells.[31,32,38] Endothelial cells, on the other hand, can enhance the proliferation and differentiation of osteoprogenitor cells by the secretion of osteogenic growth factors such as insulin growth factor-1 and endothelin-1[39] and bone morphogenic protein-2.[40–42] Moreover, it has been reported that not only growth factors, but also direct contact with endothelial cells, has a positive effect on the alkaline phosphatase activity of osteoblasts.[29,43] This study shows that in this spheroid coculture system, the addition of endothelial cells also upregulates the expression of the osteogenic marker alkaline phosphatase. Although more research is necessary to better understand the differentiation of the hMSCs in this system, our study indicates that the addition of endothelial cells is likely to have a positive effect on the osteogenic differentiation.

In summary, we have demonstrated the *in vitro* formation of a 3D prevascular network in combination with osteoprogenitor cells. Moreover, the addition of endothelial cells to hMSCs is likely to enhance the osteogenic differentiation of these cells, as was shown by the upregulation of alkaline phosphatase expression. Even though contribution of the prevascular network to the vascularization of the spheroid *in vivo* is still limited in this study, these findings indicate that *in vitro* prevascularization is a promising strategy to improve implant vascularization in the field of bone tissue engineering. This approach may also provide a tool for the *in vitro* study of bone vascularization.

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